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Sujet de la thèse : L'ACIDE DESOXYRIBONUCLEIQUE DU KINETOPLASTE
DE TRYPANOSOMA CRUZI : ORGANISATION MOLECULAIRE
ET EFFETS DE CERTAINS TRYPANOCIDES.

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Ultrastructural Alterations of *Trypanosoma cruzi* Kinetoplast Induced by the Interaction of a Trypanocidal Drug (Hydroxystilbamidine) with the Kinetoplast DNA

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The trypanocide Hydroxystilbamidine (OHSa) interacts selectively with the kinetoplast DNA (K-DNA) in *Trypanosoma cruzi*. When the drug is added to trypanosome cultures at a concentration of 5 $\mu\text{g/ml}$, it induces characteristic alterations of kinetoplast ultrastructure, not modifying any other cell component within a 7-day period. Three chronological periods occurring during drug treatment have been described: (1) Within the first 15 hours of OHSa-treatment, no ultrastructural modifications can be detected, although fluorescence shows that the drug has penetrated into the organelle. (2) Between 15 and 48 hours, the characteristic double-layered pattern of K-DNA is rearranged to form elongated fibrillar bodies. (3) During the period following 48 hours, all regular fibrillar structure disappears. The various and complex later stages are characterized by the progressive loss of the K-DNA. Dyskinetoplastic trypanosomes are formed after 6-7 days; their kinetoplast contains only a small amount of amorphous material.

The induction of "dyskinetoplasty" by drugs interacting with mitochondrial and kinetoplast DNA has been compared with the cytoplasmic "petite" mutation in yeast, which is similarly induced by the same drugs. The kinetoplast is a specialized region of the mitochondrial system of trypanosomes. It is therefore an interesting model system to study the interaction of drugs with mitochondrial DNA *in vivo*.

One of the most striking morphological features of *Trypanosoma cruzi*, a unicellular parasitic flagellate, is its large kinetoplast. The kinetoplast, situated near the basal body of the flagellum, is a part of the mitochondrial system (1, 44, 65) and contains a considerable amount of DNA (54). This kinetoplast DNA (K-DNA) seems to be particularly accessible to drugs interacting with DNA, e.g., acriflavines (11, 13, 29, 31, 34, 37, 60, 69, 71), ethidium bromide (51-53, 68, 70), berenil (3, 37, 48, 49, 57), and stilbamidines (16, 57), which are all trypanocides.

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These drugs, when added in low concentrations to the trypanosome cultures, induce selectively a progressive loss of K-DNA, leading thus to the formation of dyskinetoplastic organisms. In previous work we have tested the intercalating dye ethidium bromide (EB) (51, 52); it proved to have a specific action on the K-DNA ultrastructure (15).

Hydroxystilbamidine (OHSA) is known for its trypanocidal action (28) at the level of the K-DNA (16). The physicochemical studies concerning the interaction of this drug with DNA *in vitro* have shown that it does not intercalate between the base pairs of the DNA molecules. Its fixation is heterogeneous, related to the base composition, and selective for AT-rich DNA (21, 24).

In this work we shall describe the ultrastructural modifications induced by OHSA in the kinetoplast of *T. cruzi*.

MATERIAL AND METHODS

Trypanosome cultures. The Mexican strain Tehuantepec of *Trypanosoma cruzi* was grown in a diphasic culture medium. This strain had been kindly provided by Miss Fromentin (Institut Pasteur, Paris), and had been subcultured in this laboratory for about four years. As these cultures contain about 99% of epimastigote stages, all our observations will refer to this culture form of *T. cruzi*.

The drug was added on the first day of culture, when the population was about 5×10^5 trypanosomes per milliliter. In order to compare the growth patterns of control cultures and of trypanocide-treated cultures, cell populations were counted daily.

Drug treatment of trypanosomes for electron microscope studies. Hydroxystilbamidine (OHSA) is a stilbene diamidine compound (4,4'-diamidino-2-hydroxystilbene-diisethionate, $1 \text{ H}_2\text{O}$; from May-Baker, G. B.). In a first series of experiments, trypanosomes were exposed to different concentrations of OHSA: 2, 5, 10, 15, 20, or 30 $\mu\text{g}/\text{ml}$, and observed in the electron microscope after 2 days and 7 days. It has been found that the addition of 5 $\mu\text{g}/\text{ml}$ of OHSA to the culture medium induces morphological alterations in the kinetoplast without lethal effect on the trypanosomes within 7 days.

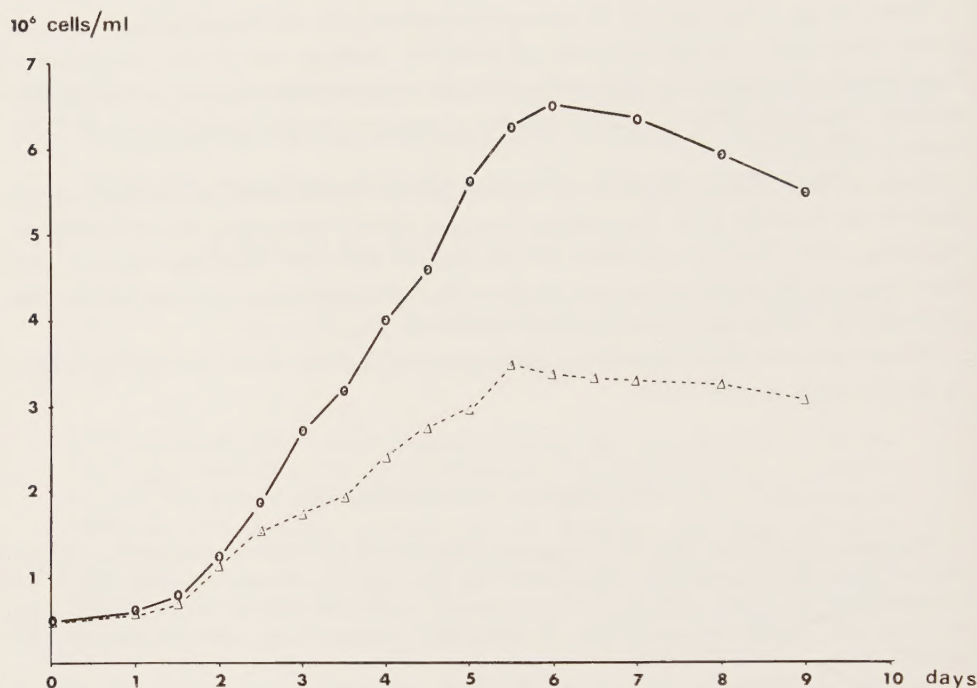
The progressive alterations of the kinetoplast ultrastructure were studied in a second series of experiments, where trypanosomes treated with 5 $\mu\text{g}/\text{ml}$ of OHSA were collected 2, 6, 15, 24, and 48 hours and 3, 4, 5, and 7 days after drug addition.

Electron microscope preparations. Most of the trypanosome cultures were processed according to the classical methods, i.e., glutaraldehyde-osmium fixation, Epon embedding, and uranyl acetate-lead citrate double staining of the thin sections.

In some cases the staining procedure described by Bernhard (4) was applied, with some minor modifications adapted to our material. This method allows preferential staining of ribonucleoprotein-rich cell components, while DNA is unstained by EDTA, which preferentially removes the uranium stain linked to DNA.

Trypanosomes are fixed only with glutaraldehyde, 1 hour at 4°C , Sørensen phosphate buffer 0.1 M, pH 7.2.

The thin sections (previously selected of uniform thickness) are then treated as follows:



The scale on all photographs represents 0.5 μm .

FIG. 1. Growth patterns of *Trypanosoma cruzi* cultures. $\circ-\circ$, Control culture with an exponential phase between 2nd and 6th day; $\triangle-\triangle$, growth curve of a culture containing 5 $\mu\text{g/ml}$ OHSA. The growth rate between the 2nd and 6th day is decreased.

—prestaining with aqueous uranyl acetate 5%, 2 minutes

—treatment with EDTA 0.02 M, aqueous solution, 1–5 minutes (technical EDTA, Fluka, tetrasodical salt)

—poststaining with Reynolds' lead citrate, 1 minute.

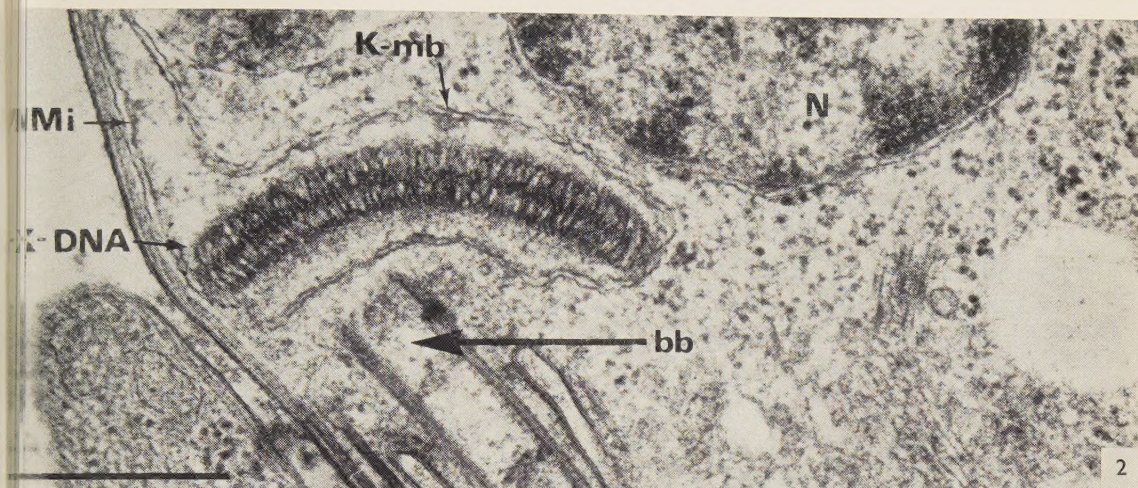
For control preparations, glutaraldehyde fixed specimens were stained with uranyl acetate 5% for 2 minutes, followed by lead citrate 1 minute. Photographs were taken with a Philips EM 300.

FIG. 2. Untreated trypanosome showing the ultrastructure of the kinetoplast situated between the basal body of the flagellum (*bb*) and the nucleus (*N*). The K-DNA is arranged in a characteristic double-layered organization. Note the continuity of the kinetoplast membrane (*K-mb*) with the mitochondrial membrane (*Mi*). $\times 54\,000$.

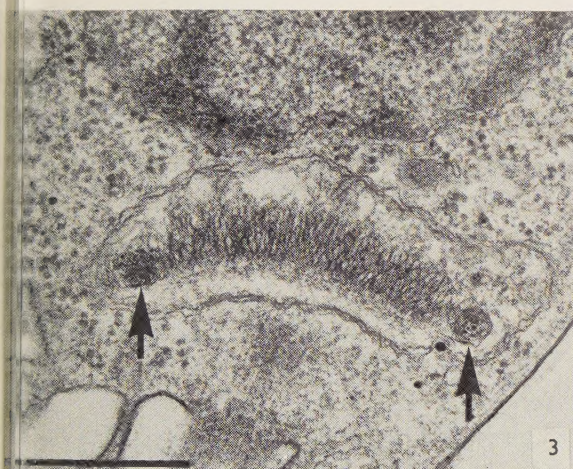
FIG. 3. Early K-DNA alterations in trypanosomes treated for 15 hours with 5 $\mu\text{g/ml}$ of OHSA. Appearance of spherical fibrillar bodies ($\rightarrow$) at the periphery of the K-DNA. $\times 43\,000$.

FIG. 4. Progressive reorganization of the fibrillar material into spherical bodies in one or two overlaid rows. OHSA, 5 $\mu\text{g/ml}$, 48 hours. $\times 54\,000$.

FIG. 5a–c. Transverse serial sections show that the spherical structures are in fact cross sections of elongated cylindrical bodies. OHSA, 5 $\mu\text{g/ml}$, 48 hours. $\times 54\,000$.



2



3



4



5a



5b



5c

RESULTS

Trypanosome cultures

Fig. 1 shows the growth curves of a control culture and of a culture of *T. cruzi* treated with OHSA (5 μ g/ml). During the adaptation period of 1–2 days very few cell divisions are observed. The control cultures then pass to the exponential phase (2nd–6th day) and the population doubles once every 24 hours. The drug-treated trypanosomes, however, multiply at a slower rate, their number doubling every 48 hours. At the end of the exponential phase, i.e., in 6- or 7-day-old cultures, about 15–20% of the population are dyskinetoplastic trypanosomes. Such trypanosomes have no visible kinetoplast in Giemsa-stained preparations.

Morphology of untreated trypanosome kinetoplast

In the epimastigote form of *T. cruzi* the kinetoplast is an arched disk-shaped organelle, the concave side facing the basal body of the flagellum. In transverse sections through the kinetoplast (i.e., sections parallel to the flagellar axis), the K-DNA appears to be arranged into a very regular and characteristic double-layered pattern (Fig. 2) (8, 14, 38). Horizontal sections through the kinetoplast (i.e., sections perpendicular to the flagellar axis) show the cross-sectioned K-DNA filaments as tiny, randomly dispersed dots.

Ultrastructural alterations

1. During the first 15 hours of OHSA treatment, the kinetoplast shows no alterations.

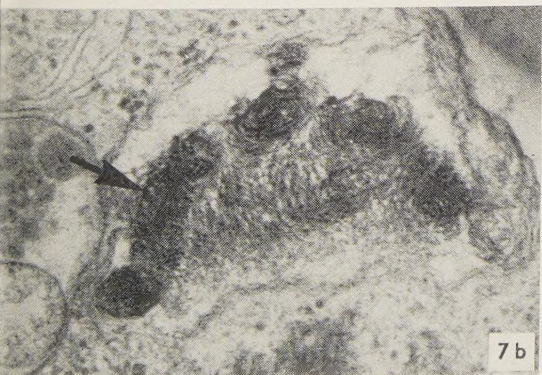
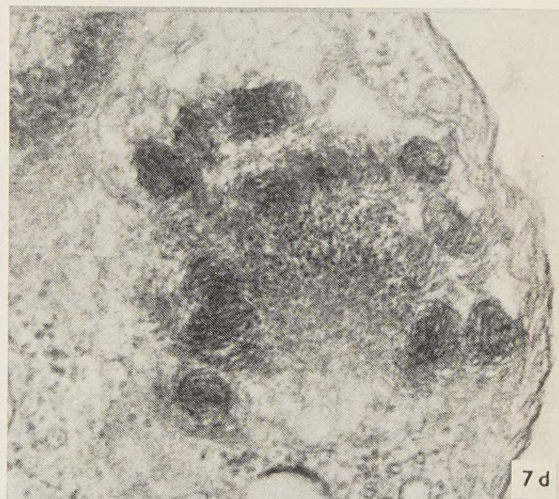
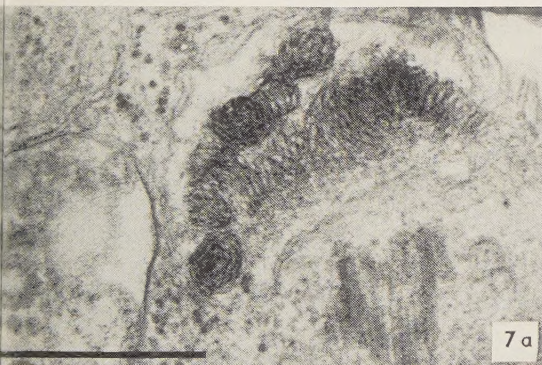
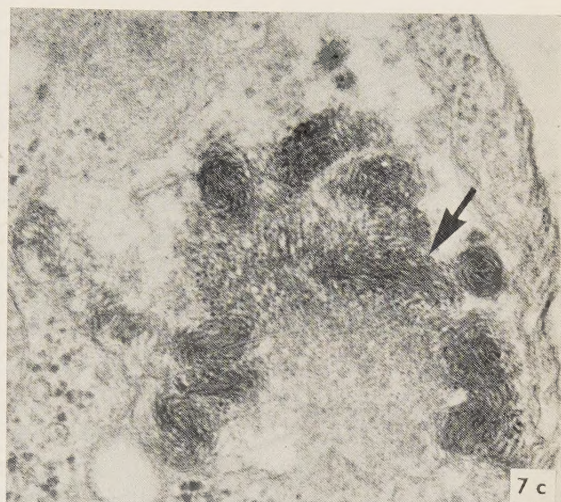
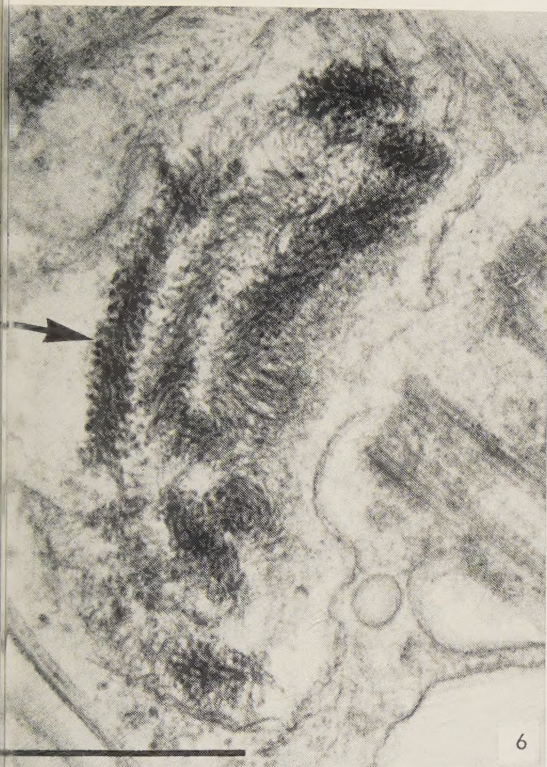
2a. Between 15 and 48 hours of treatment, spherical bodies appear at the edges of the DNA disk (Fig. 3, $\rightarrow$). They consist of peripheral fibrillar material surrounding a central zone of dense dots (Figs. 3 and 4).

2b. In later stages (48 hours OHSA), several fibrillar spheres are grouped either in a single or in a double row (Fig. 4). The reorganization of the fibrous structure of the K-DNA seems to progress from the periphery toward the center of the kinetoplast. As illustrated in Figs. 4 and 5, the normal regular arrangement of the K-DNA is still maintained in the central region.

In order to determine the actual three-dimensional shape of the spherical bodies, serial sections were made. Fig. 5 shows that the spherical structures are similarly

FIG. 6. Transverse section at the periphery of the kinetoplast. The fibrillar material is organized into elongated, "sausage-shaped bodies" ($\rightarrow$). OHSA, 5 μ g/ml, 48 hours. $\times 65\,000$.

FIG. 7a–e. Five successive serial sections of one kinetoplast show the complex imbrication of the cylindrical fibrillar bodies ($\rightarrow$). In Fig. 7e the "sausages" are at the periphery, surrounding the still unmodified central DNA zone. OHSA, 5 μ g/ml, 48 hours. $\times 54\,000$.



arranged in two adjacent serial sections (Figs. 5a, b) and only slightly differently in a nonadjacent section (Fig. 5c). We can conclude that they may be cross sections through cylindrical fibrillar bodies or through a row of fibrillar spheres.

Fig. 6 represents a transverse section, tangential to the edge of the kinetoplast, which shows that the fibrillar bodies are elongated and preferentially situated at the periphery of the K-DNA disk. In horizontal or slightly oblique serial sections (Figs. 7a-e), we can see several imbricated cylindrical structures. The central dotted zone (Fig. 7e) corresponds to the normal arrangement of cross-sectioned DNA-filaments. Figs. 6 and 7 also show some further details of the arrangement of the DNA within the "sausage-shaped" bodies: the central region contains long filaments which are parallel to the axis of the elongated body ($\rightarrow$).

2c. Some more advanced stages of kinetoplast alteration appear on the 2nd and 3rd day of OHSA treatment (Figs. 8 and 9). They consist in an amplification of the phenomenon: complete reorganization of the K-DNA into cylindrical fibrillar structures (Fig. 8). On the right of the kinetoplast of Figs. 9a, b, c, d, in four successive serial thin sections, the "sausages" are oriented perpendicularly to the section plane and therefore have a similar appearance. The elongated bodies on the left are either longitudinally or cross sectioned, according to their oblique orientation (Figs. 9a-g).

3. After 3 days of treatment, the regular fibrillar structures disappear, leaving a disorganized DNA mass with clear zones (Fig. 10). They seem to be cavities and contain some very thin filaments (approximately 25 Å, ($\rightarrow$) Fig. 10). In addition, some small electron dense particles appear at the periphery of the DNA.

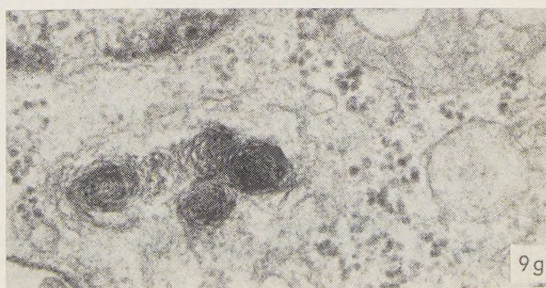
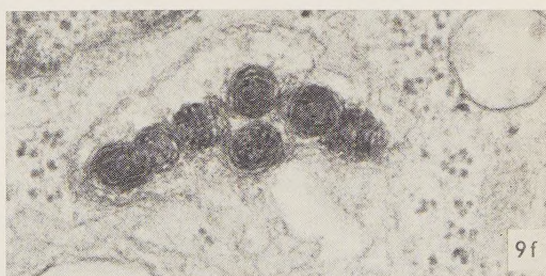
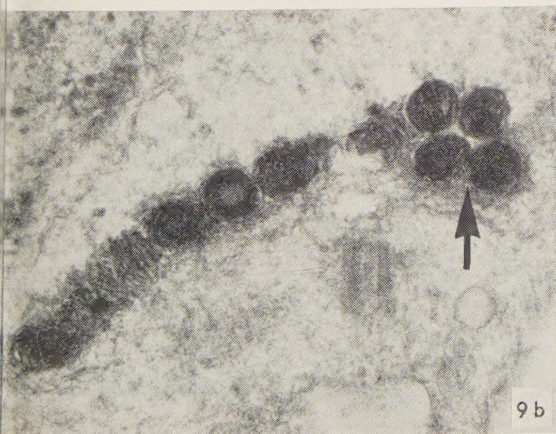
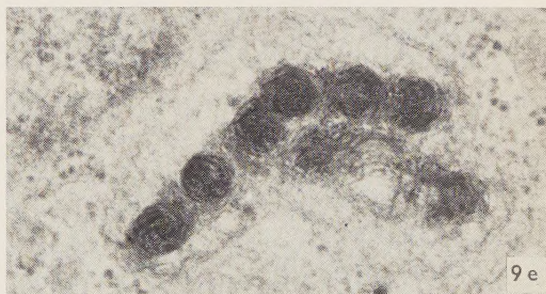
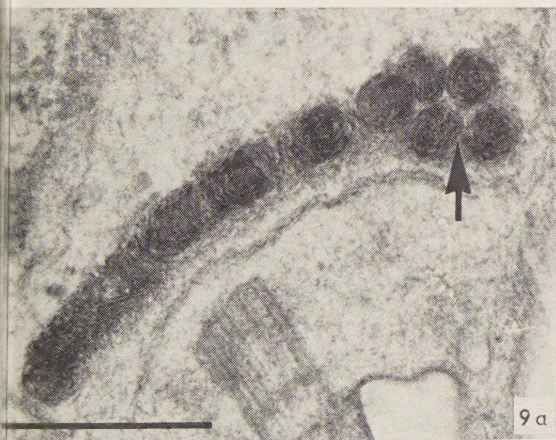
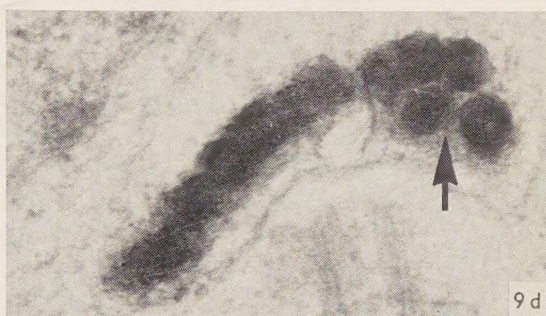
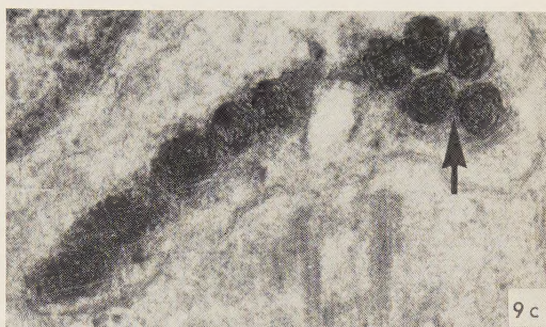
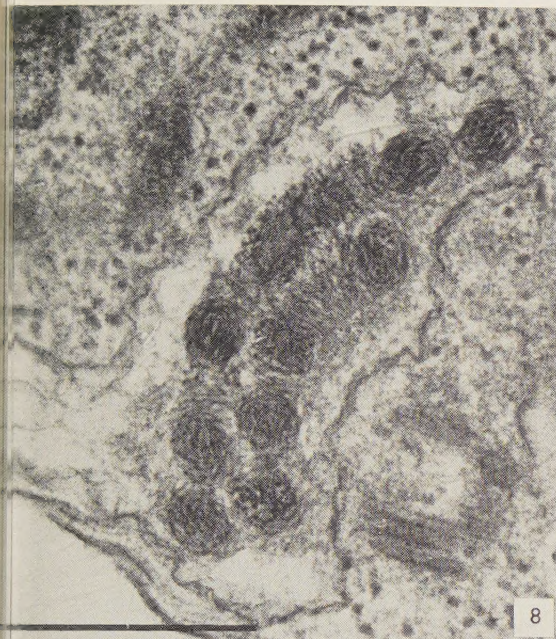
Another aspect of the loss of fibrillar organization is illustrated in Fig. 11. The disk shape of the kinetoplast is only partially conserved; one part of the DNA is completely disorganized into irregular masses of different electron density. In such stages the kinetoplast membrane is often swollen (Fig. 11).

When the trypanosomes are treated for 4-7 days with 5 μ g/ml of OHSA, or for shorter periods with higher drug concentrations (10-30 μ g/ml), very dense irregular material can be seen instead of the fibrillar K-DNA (Fig. 12, black $\rightarrow$). Masses of fine granular material persist at the periphery (Fig. 12, white $\rightarrow$). Fig. 12 shows a dividing kinetoplast; one of the daughter-kinetoplasts (a), contains the modified DNA, the other one (b) contains some granular material only. Such a picture suggests the way dyskinetoplastic trypanosomes are formed.

In Fig. 13 the central core of the kinetoplast has no electron density, except for

FIG. 8. All the K-DNA is now reorganized into two parallel layers of fibrillar bodies. OHSA, 5 μ g/ml, 48 hours. $\times 65\ 000$.

FIG. 9a-g. Transverse serial sections favorably oriented to allow a three-dimensional reconstruction. See for example, the continuity of the four cylindrical bodies which are cross-sectioned on the right of the kinetoplast (a-d, $\rightarrow$). OHSA, 5 μ g/ml, 72 hours. $\times 54\ 000$.



the peripheral zones which consist of irregular dark granules (black→). Either extremity contains the gray amorphous material (white→). The cavities are crossed by thin trabeculae and filaments.

With the staining procedure described by Bernhard, DNA containing structures are preferentially unstained. We have applied this technique in order to detect the presence of DNA in the complex structures of modified kinetoplasts.

In normal trypanosomes the K-DNA is unstained after 2 minutes of contact with EDTA (Fig. 14, white→), while the nuclear chromatin still remains contrasted (Fig. 14, *N*). When the sections of OHSA-treated trypanosomes are equally unstained for 2 minutes, it is possible to distinguish between the slightly modified K-DNA, which is unstained as rapidly as normal K-DNA (Fig. 15, white→), and dark material which is not bleached (Fig. 15, black→). Three minutes of EDTA are necessary to unstain nuclear deoxyribonucleoproteins (Fig. 16, *N*). The kinetoplast content is unstained as well (white→), except for the dark masses (black→). When the EDTA procedure is prolonged up to 5 minutes, almost all cell components, including the previously still contrasted regions of the altered kinetoplast, are unstained. Only the ribonucleoprotein-rich regions in the cytoplasm and in the nucleus are clearly distinguishable (Fig. 17).

Figs. 18 and 19 show some OHSA-treated trypanosomes with further interesting features. In some trypanosomes with a modified kinetoplast, the posterior part of the mitochondrion is swollen and contains granular and electron-dense material (Figs. 18a and b), which is similar to the dense accumulations that we described for Fig. 11. In cultures treated for 5–7 days with OHSA, abnormal divisions were frequently observed. The kinetoplast elongates, but the nondividing K-DNA keeps the two daughter-cells attached to each other, whereas the nuclear division and cytokinesis proceed normally (Fig. 19).

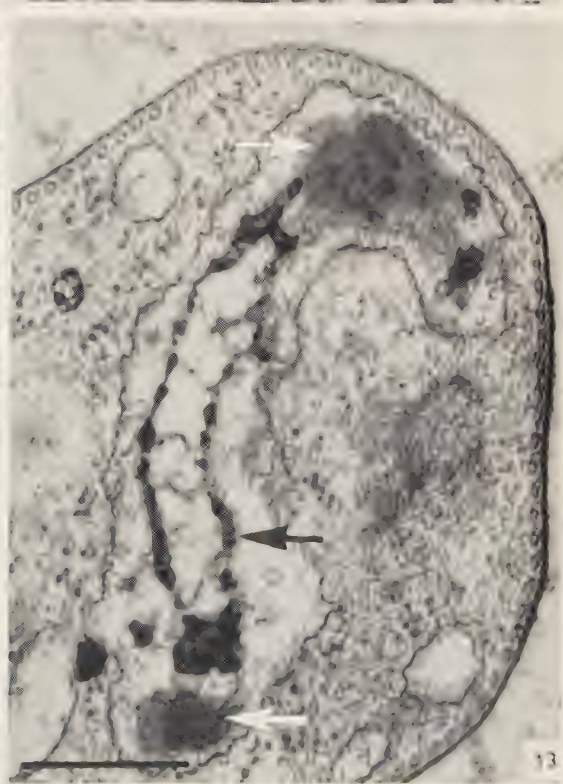
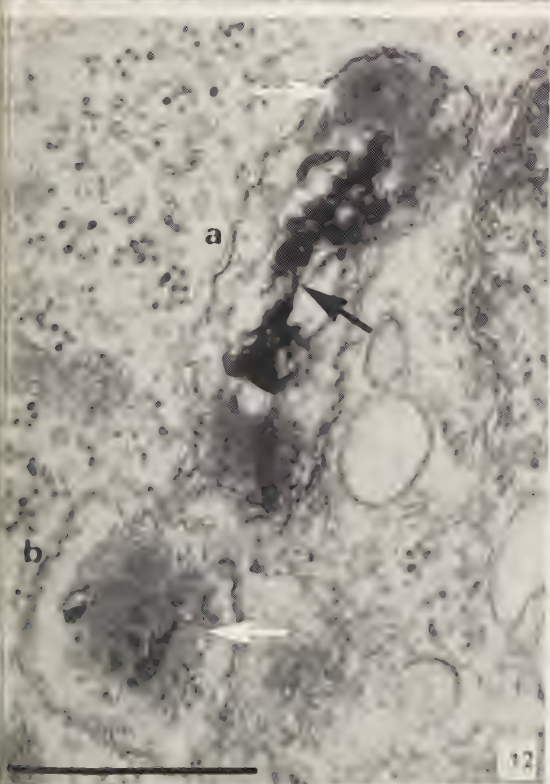
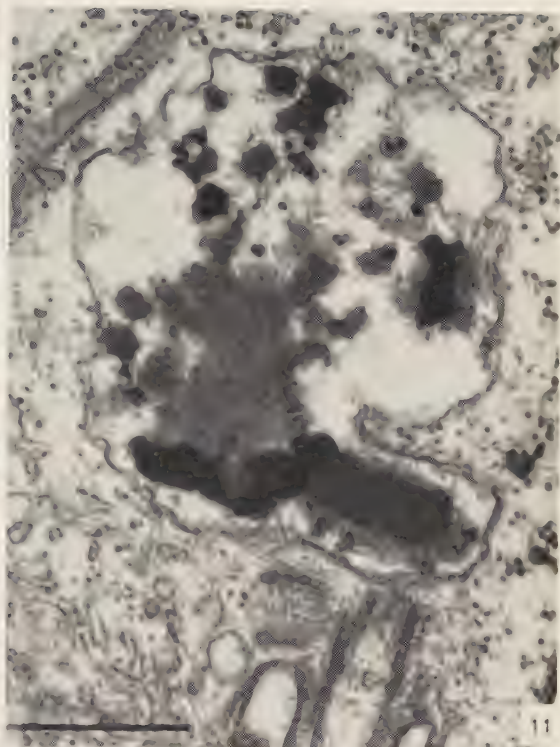
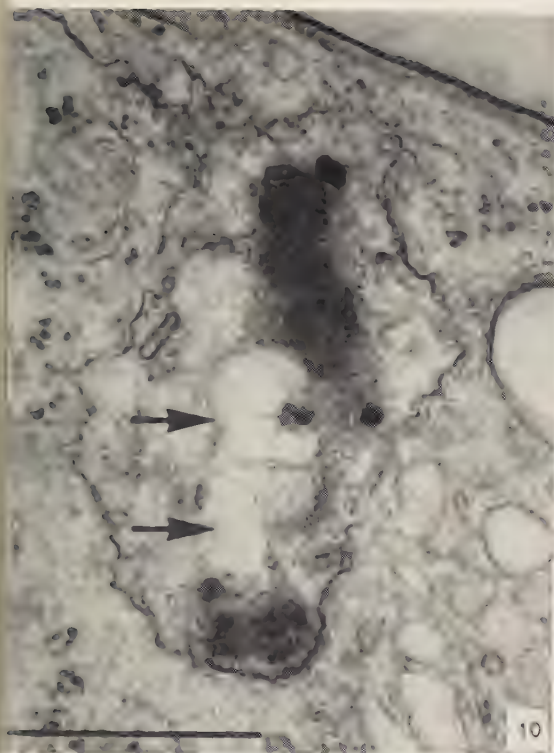
Finally, some trypanosomes have a very reduced granular kinetoplast content (Fig. 20), which is not visible in Giemsa-stained preparations: the trypanosomes are then called dyskinetoplastic.

FIG. 10. Disappearance of the regular fibrillar DNA structures. Cavities in the DNA-disk contain about 25 Å filaments (→). OHSA, 5 µg/ml, 72 hours. × 65 000.

FIG. 11. The kinetoplast membrane is swollen, filled with irregular granular material of various electron density. All organized fibrillar structure has disappeared. OHSA, 5 µg/ml, 5 days. × 43 000.

FIG. 12. Dividing modified kinetoplast. One daughter-kinetoplast (*a*) contains very dense material (black→) and gray granular masses at its periphery (white→); the other one (*b*) contains only some granular material (white→). OHSA, 15 µg/ml, 3 days. × 65 000.

FIG. 13. An electron-clear zone is surrounded by dark material (black→) and crossed by filaments (25 Å) and trabeculae. The gray granular material at the periphery is similar to that seen in Fig. 12 and in dyskinetoplastic trypanosomes (Fig. 20). OHSA, 30 µg/ml, 5 days. × 43 000.



DISCUSSION

Many drugs interacting with DNA, such as acridines [e.g., acriflavine (73), proflavine (36)], phenanthridines [e.g., ethidium bromide (35, 72, 73)], and diamidines [e.g., berenil (48, 49, 73), hydroxystilbamidine (21, 24)] are known to be trypanocides. These substances produce various morphological alterations in the kinetoplast (15, 16, 60, 69).

In this paper we have shown the progressive ultrastructural changes induced by OHSA in the kinetoplast of *T. cruzi*, which finally lead to the formation of dyskinetoplastic trypanosomes. They are not able to divide in culture and their kinetoplast contains a reduced amount of DNA.

"Dyskinetoplasty" (41) is also a naturally occurring phenomenon: dyskinetoplastic strains of *T. equiperdum*, *T. equinum*, and *T. evansi* have been described. Although their kinetoplast does not contain a detectable amount of K-DNA, they are viable in their vertebrate host, but cannot be maintained in culture (43). Thus "dyskinetoplasty" is both natural and induced by drugs; this fact reminds us of the "petite" or *ρ* cytoplasmic mutation of yeast (18–20), as previously mentioned (71). This mutation, expressed by the disappearance of mitochondrial respiratory enzymes, has its origin in the mitochondrial DNA (M-DNA) (27). It is also natural or induced by drugs (acridines: 19, 63; EB: 62; OHSA: 42), as well as by a number of other agents (45, 74). These drugs preferentially interact with cytoplasmic nucleic acids and alter their metabolism; as seen in yeast mitochondrial DNA, trypanosome kinetoplast DNA and prokaryote extrachromosomal DNA. Considering an analogy between M-DNA and K-DNA, the trypanosome serves as a model system to study the drug interaction with mitochondrial DNA. M-DNA and K-DNA are both free of histones (46, 66, 74), resembling in this respect to prokaryote DNA, and seem to be particularly accessible to drugs.

Among the above-mentioned DNA-interacting drugs, two classes can be distinguished: (a) dyes intercalating between the base pairs of the DNA double helix,

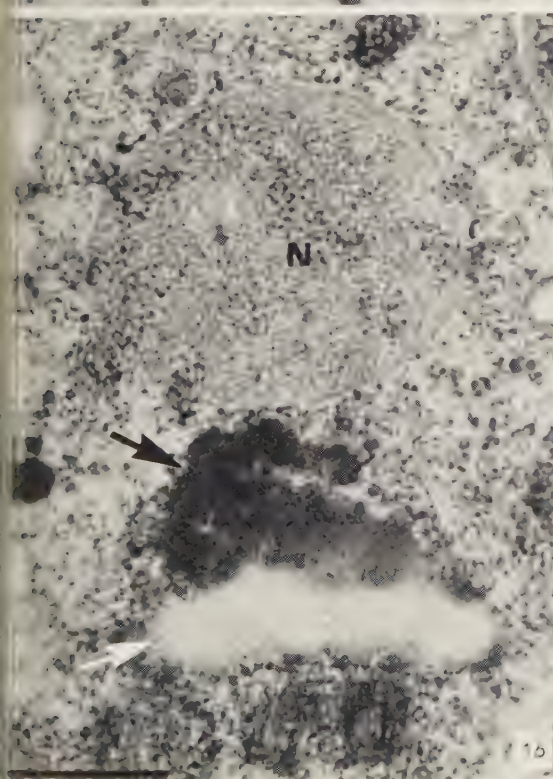
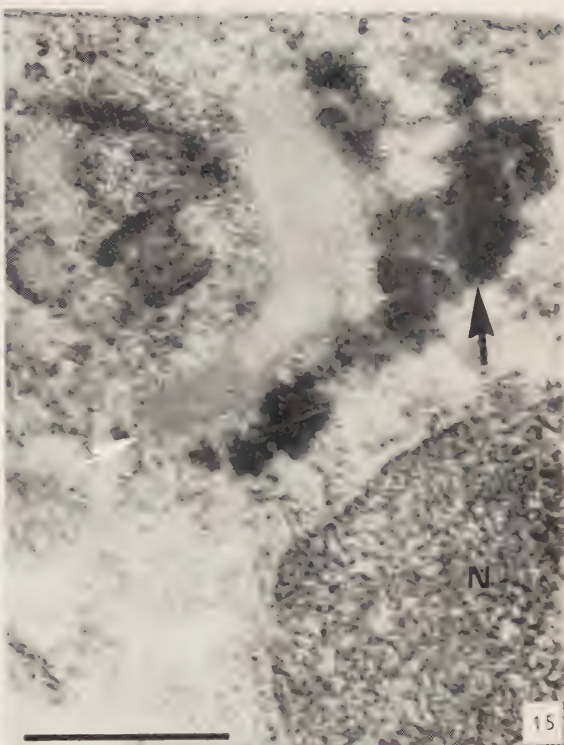
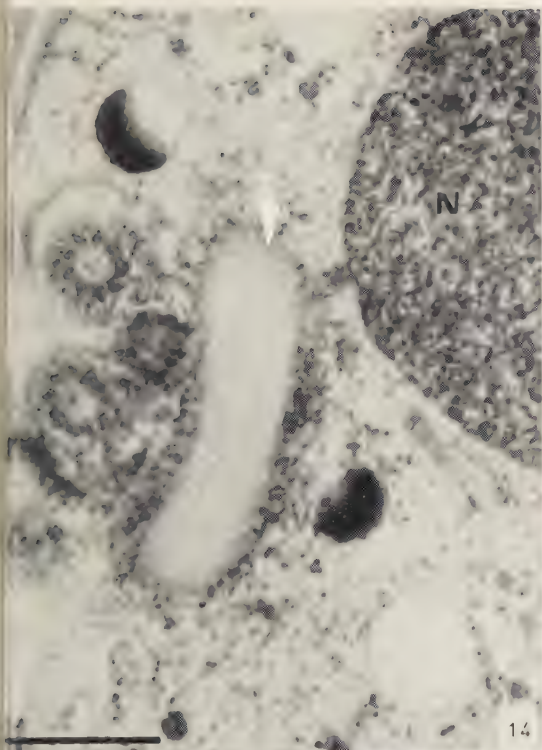
FIGS. 14–17. All preparations are fixed only with glutaraldehyde and treated with EDTA.

FIG. 14. Trypanosomes of a control culture (without OHSA) bleached by 2 minutes EDTA. The normal K-DNA is completely unstained (white→), while nuclear chromatin is still contrasted (N). $\times 41\ 000$.

FIG. 15. With the same EDTA treatment (2 minutes) the slightly modified K-DNA (white→) is unstained, but the dense irregular material (black→) is not. OHSA, 10 $\mu\text{g/ml}$, 6 days. $\times 54\ 000$.

FIG. 16. The nuclear deoxyribonucleoproteins are unstained after 3 minutes EDTA. In contrast to the modified K-DNA (white→), the accumulated black material still resists to the unstaining procedure (black→). OHSA, 10 $\mu\text{g/ml}$, 6 days. $\times 41\ 000$.

FIG. 17. A 5-minute EDTA treatment unstains all cell components, even the material accumulated in the kinetoplast (white→), except for ribosomes and nuclear RNP. OHSA, 10 $\mu\text{g/ml}$, 5 days. $\times 54\ 000$.



e.g., acridines (36) and EB (35, 72); (b) drugs which do not intercalate, but show a preferential binding to AT-rich DNA, e.g., OHSA (21) and berenil (22–24). This property might account for their specific binding to the K-DNA of *T. cruzi*, which is rich in AT (54).

Three types of interaction of these drugs with DNA can be proposed:

1. The intercalating drugs have a *direct action* on DNA conformation, modifying the tertiary structure of the DNA molecules (35, 36, 72, 73). The insertion of dye molecules produces a torsion of the double helix, modifying the supercoiling; this might introduce mistakes during replication and transcription by a frame-shift mechanism (9).

2. An *indirect action* can be considered at the level of the enzymes of nucleic acid metabolism. A specific action on extranuclear DNA would then result from a distinction between mitochondrial and nuclear enzymes (32). A number of recent papers have actually shown such a difference between mitochondrial and nuclear enzymes implied in nucleic acid metabolism. Meyer and Simpson have specified (39) and purified (40) a specific mitochondrial DNA polymerase from rat liver; this enzyme is selectively inhibited by EB, which does not affect the nuclear DNA polymerase. Also mitochondrial RNA synthesis is specifically inhibited by EB and acridines and other agents in a wide range of organisms: yeast (25), rat liver (26), HeLa cells (33, 75). These drugs further interact with tRNA and may thus disturb protein synthesis (5).

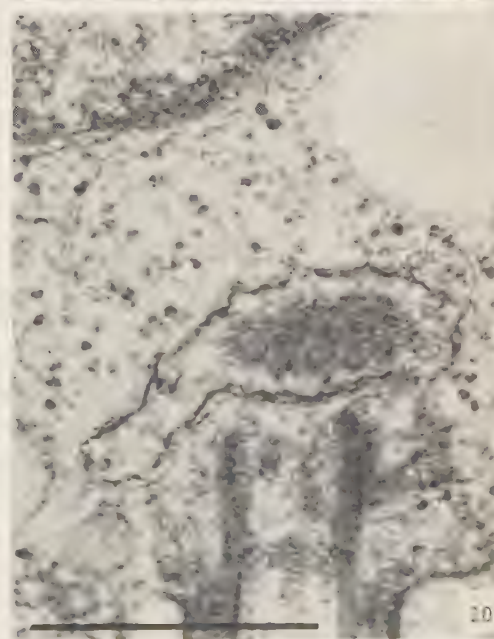
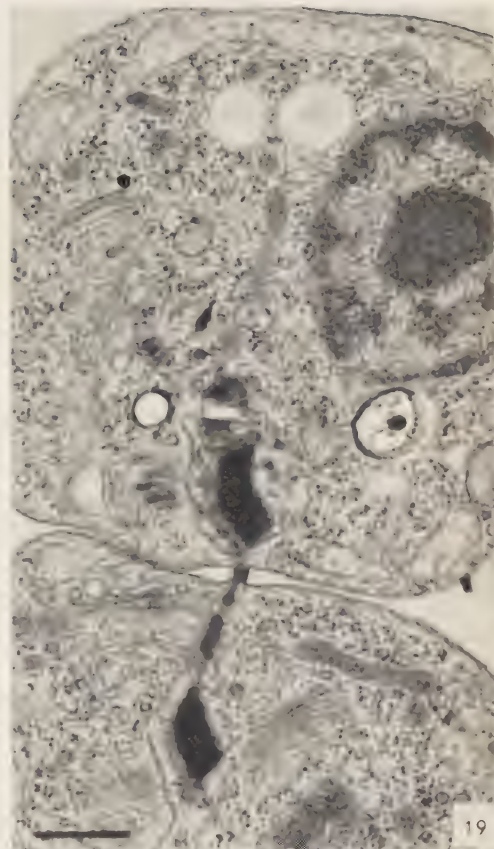
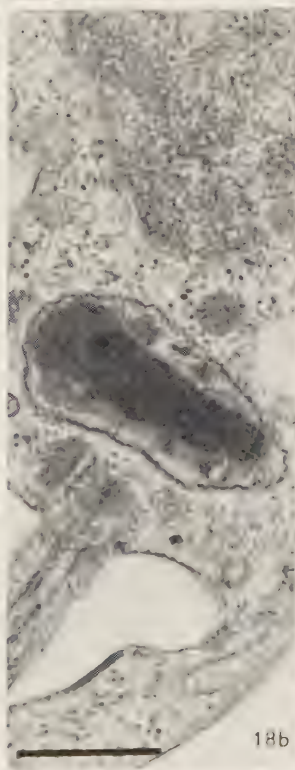
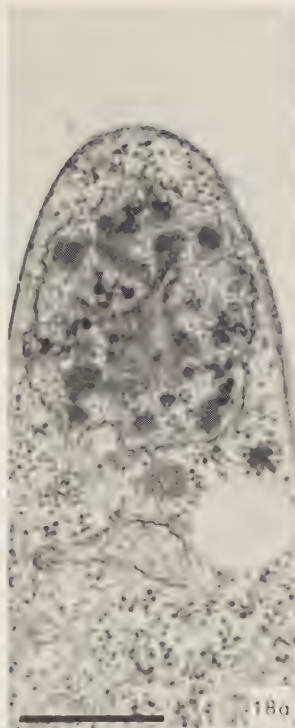
3. Another target of indirect action could be the mitochondrial respiratory enzymes. As mentioned above, the “petite” yeast lack respiratory enzymes. Trypanosomes treated with acriflavine or EB show a decrease in respiratory enzymes and an increase in glycolytic enzymes (29, 30, 34). In rat liver mitochondria, EB and euflavine exert a specific inhibitory effect on the enzyme cytochrome *c* oxidase (17).

In order to study the interaction of trypanocidal drugs with the K-DNA and to understand the chronology of the lesions produced by these drugs, it would seem logical to perform experiments with synchronously dividing organisms. Steinert et al., and Simpson have obtained synchronized division of *Trypanosoma mega* (67) and *Leishmania tarentolae* (61), respectively, with hydroxyurea. We were not able to get any satisfactory results with our material. Furthermore, a synchronization obtained by blocking the nucleic acid metabolism by hydroxyurea was not a good method for us, when the effect of DNA-interacting drugs would be studied afterward. It has been

FIG. 18. Electron dense material accumulates in the mitochondrion at the posterior end of the trypanosome. (a) and (b) show enlarged regions of the mitochondrion and the modified kinetoplast. OHSA, 5 μ g/ml, 5 days. $\times 18\,000$; (a, b). $\times 37\,000$.

FIG. 19. Abnormal cell division: the modified K-DNA, which is unable to divide prevents the separation of the two daughter cells. OHSA, 5 μ g/ml, 3 days. $\times 27\,000$.

FIG. 20. Kinetoplast of a dyskinetoplastic organism. It contains no fibrillar regular structure, only a small amount of gray amorphous material. OHSA, 5 μ g/ml, 7 days. $\times 76\,000$.



shown that hydroxyurea does actually interfere with various enzymes implied in DNA synthesis (12, 50).

For each treatment period the most important modifications were grouped according to their relative frequency. This led to a classification of our results into three successive periods, which, of course, can only be approximately delimited.

1. Within the first 15 hours of OHSA treatment, we have not been able to detect any morphological alterations in the kinetoplast. However, OHSA seems to be rapidly incorporated into the organelle. UV-microscope observations show an intense fluorescence of the kinetoplast shortly after the drug is added to the culture; this is due to the enhanced fluorescence of the dye when it binds to DNA (21). It is possible that the following alterations of the DNA structure are not a direct consequence of the OHSA-DNA interaction.

2. The period between the 2nd and the 3rd day of drug treatment is characterized by the progressive modification of K-DNA. The regular pattern of K-DNA is rearranged into elongated fibrillar structures ("sausage-like bodies").

Owing to its high concentration, the K-DNA may have a paracrystalline conformation (7). It has actually been shown that DNA (58) and tRNA (64) show properties similar to liquid crystals. The EB-induced modifications (arches) described in an earlier paper (15) had been suggested to be due to the intercalating properties of the drug, which modifies the tertiary structure of covalently closed circular DNA. These alterations seem to be another aspect of the mesomorphous state of the K-DNA. But as OHSA is not an intercalating dye, it has no effect on the tertiary structure of the DNA molecules (24). Therefore, the rearrangement of K-DNA described here ("sausage-like structures") must be explained by another mechanism.

Two possibilities can be suggested: as the drug molecules penetrate between the tightly packed K-DNA molecules, they may change the lattice structure of the liquid crystal. On the other hand, OHSA may block the enzymes implied in DNA replication. In experiments that will be described elsewhere, we have compared the DNA of isolated, osmotically lysed kinetoplasts of untreated and OHSA-treated trypanosomes. In treated trypanosomes the K-DNA molecules are packed in such a way as to suggest that their replication had been blocked.

The "sausage-like bodies" always appear first at the periphery of the kinetoplast; the modifications then proceed toward the center, until the whole K-DNA is in the form of imbricated cylindrical bodies. When trypanosomes were incubated for a short time with thymidine- H^3 , autoradiograms show sometimes a preferential labeling at the periphery of the DNA disk. This finding is in accordance with the results of other authors (2, 10). If thymidine- H^3 is first incorporated at the peripheral zones, it can be assumed that K-DNA replication begins in these regions. The drug action may therefore start in these metabolically active zones.

3. In the third period, i.e., after 3 days of 5 μ g/ml OHSA, or earlier with higher concentrations, there is a great diversity of stages. The most characteristic feature is the complete disappearance of all fibrillar organization of the K-DNA. The residual amorphous material may still contain DNA, but not enough for the characteristic paracrystalline state of the normal kinetoplast DNA.

Goldring has proposed that the distortion of supercoiled molecules by EB intercalation may lead to increased nuclease attack (27). As the paracrystalline array of K-DNA is probably disturbed by OHSA, it is possible to think that nucleases can more easily attack modified K-DNA, leading to the formation of cavities.

Two mechanisms have been proposed to explain the progressive loss of K-DNA during acriflavine treatment and can be applied to OHSA-induced "dyskinetoplasty": (a) progressive dilution of the K-DNA by inhibition of its replication (43, 71); (b) an unequal distribution of K-DNA may finally yield one "normal" and one dyskinetoplastic cell (11, 59, 60).

In fact, the two phenomena may occur simultaneously.

The ultrastructural aspect of the residual kinetoplast content in OHSA- or EB-induced dyskinetoplastic trypanosomes is similar to that of normally dyskinetoplastic cells (41).

Bernhard's staining procedure with EDTA gives us further possibilities of investigation. The dense material accumulated in kinetoplasts and mitochondria does not bleach as rapidly as normal or slightly modified K-DNA, nor as nuclear deoxyribonucleoproteins. The dense masses are either a OHSA-K-DNA complex insensitive to the EDTA action, or accumulations of mitochondrial proteins.

The extranuclear DNA of *T. cruzi* consists of two populations of molecules (54). The first one with a buoyant density of $\rho = 1.699$ g/ml is composed mainly of covalently closed circular duplexes (0.45 μ) and some long linear molecules. The second population ($\rho = 1.686$ g/ml) consists of linear molecules and is more or less abundant in our cultures (55). The treatment of trypanosomes with OHSA induces the disappearance of the circular molecules and the formation of complex molecular associations. It is interesting to note, that there were no abnormal molecules (oligomers) such as induced by EB treatment (56). The two mechanisms of molecular interaction of OHSA and EB with DNA may account for this difference.

We have paralleled OHSA with other drugs interacting with extranuclear DNA, inducing "dyskinetoplasty" in trypanosomes and the "petite" mutation in yeast. During the first 48 hours of incubation with each respective drug, the ultrastructural as well as the molecular modifications are different, depending on the use of intercalating and nonintercalating drugs. But in both cases the end result is the loss of mitochondrial or kinetoplast DNA. The final stage in trypanosomes, i.e., "dyskinetoplasty", is not specifically related to the mode of molecular interaction of the drug

with K-DNA. The fact, that the observed intermediate stages are different, suggests the existence of various distinct targets at the level of mitochondrial metabolism. Mitochondria of higher organisms contain only a small amount of M-DNA (6). In contrast, trypanosomes contain a large amount of M-DNA in their kinetoplast. Therefore, we consider the trypanosome to be a very interesting model system for studying the interaction of M-DNA with drugs *in vivo*.

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